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Association Between Historically High Frequencies of Neural Tube Defects and the Human *T* Homologue of Mouse *T (Brachyury)*

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The human *T* developmental gene has been implicated in the etiology of neural tube defects (NTDs) on the basis both of mouse studies of its homologue, *T (Brachyury)*, and of allelic association in a Caucasian population. We have investigated the frequency of the *T* allelic variant *TIVS7-2* in 218 Irish NTD case-parent triads. This population showed the same trend as previously reported, with an excess of the *TIVS7-2* allele among cases. Log-linear modeling of case and maternal genotypic effects within families indicated that *TIVS7-2* was elevated in cases (relative risk, RR = 1.36) but not in mothers (RR = 0.91). The *TIVS7-2* allele is markedly associated with cases born before 1980 (RR = 2.09; CI = 1.23–3.55; corrected p = 0.030), but not with more recent cases (RR = 0.92). Cases carrying a *TIVS7-2* allele did not show any increased tendency to be homozygous for the thermolabile variant of the folate-dependent enzyme 5,10-methylene tetrahydrofolate reductase, which is an established genetic risk factor for NTDs. Since the incidence of NTDs has declined markedly in Ireland over the last few decades, we suggest that the *T*-associated risk is potentiated by nutritional or environmental risk factor(s), the impact of which have been diminishing over time. Am. J. Med. Genet. 92:206–211, 2000.

KEY WORDS: genetics; folate; MTHFR; spina bifida

INTRODUCTION

Neural tube defects (NTDs), in particular spina bifida and anencephaly, are relatively common severe congenital malformations. Although not fully understood, the etiology of NTDs is generally considered to involve a range of predisposing genetic factors [Little and Nevin, 1992]. The observation that women who have already given birth to an affected child have a 10–15-fold increased risk that a subsequent child will have an NTD [Little, 1992] reinforces the view that there is a strong maternal and/or familial pathogenic component. Nutritional and possibly other environmental factors also contribute to NTD outcome. Several studies over the past decade have established that folic acid supplements taken periconceptionally can reduce a woman's risk of both NTD occurrence and recurrence by up to 70% [Czeizel and Dudas, 1992; MRC Vitamin Study Research Group, 1991]. It is now known that women carrying NTD fetuses often have significantly reduced plasma folate and vitamin B12 concentrations that are accompanied by significantly elevated plasma levels of the amino acid homocysteine [Kirke et al., 1993; Mills et al., 1995].

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One of the enzymes that participates in the control of plasma homocysteine concentrations is folate dependent 5,10-methylenetetrahydrofolate reductase (MTHFR). Individuals who are homozygous for the "thermolabile" form of MTHFR, which is mildly dysfunctional and is associated with increased plasma homocysteine concentrations under conditions of low folate [Harmon et al., 1996; Jacques et al., 1996], comprise around 8% of the Irish population (and between 5 and 16% of most other Caucasian populations). An obvious candidate genetic risk factor for NTDs, the homozygous thermolabile MTHFR genotype has been shown in case-control studies to be associated with NTD pregnancies in the Irish [Whitehead et al., 1995] and Dutch [van der Put et al., 1995] populations. A subsequent study of mothers of NTD cases suggested that folate status, rather than MTHFR genotype, is the critical maternal component [Molloy et al., 1998], and recently we have established that it is in the embryo that the pathogenic potential of the homozygous thermolabile MTHFR genotype is realized [Shields et al., 1999].

We have estimated that only 12% of NTDs are attributable to the homozygous thermolabile MTHFR genotype [Shields et al., 1999], or to associated alleles at the locus. Therefore, the more than 50% of all folate-preventable cases that are not MTHFR-related may be partly caused by failures in other, as yet unidentified, folate-dependent metabolic or developmental pathways. In addition, the possibly folate independent etiologic mechanism(s) that underlies the balance of NTDs, comprising around 30% of cases, is also unknown.

Neurulation is a multifactorial process involving neural plate shaping and changes in cell behavior that is governed by a series of secreted signaling molecules, receptors, and transcription factors [Smith and Schoenwolf, 1997]. Data from mouse mutants exhibiting neural tube defects suggest that extrinsic signals are important for neural plate folding and neural groove closure. Some of these inductive processes occur between the neural tube and the underlying notochord. Morrison et al. [1996] have reported that an allelic variant of the *T (Brachyury)* locus shows a bias in transmission from heterozygous parents to NTD cases in Dutch and U.K. families, and may be a risk factor for this group of abnormalities. T is a transcription factor expressed at high levels in the notochord during neurulation and is essential for normal axial development in vertebrates. Mice that are homozygous for loss of function mutations in the equivalent locus *T (Brachyury)* die in midgestation with severe defects in posterior mesodermal tissues; heterozygous mice have shortened or nonexistent tails, lack of separation between gut and neural tube, and occasional malformations of the sacral vertebrae [Wilson et al., 1993]. The observations of Morrison et al. [1996] with respect to *T* locus-associated NTD risk are therefore compatible both with the biology of the *T* locus product and the developmental phenotype elicited by mutants thereof in an animal model. Trembath et al. [1999] failed to find excessive transmission of *TIVS7-2* to meningomyelocele patients, but there were only 21 heterozygous parents to test transmission bias.

In this study we have confirmed the involvement of a *T* locus variant (or alleles in disequilibrium with it) in human NTDs by analyzing the segregation of the *TIVS7-2* allele among 218 Irish case-parent triads. However, there is no evidence for a pathogenic interaction between the *T* locus mutation and the thermolabile MTHFR genotype, a known risk factor for NTDs. In addition, we have established that the proportional contribution of the *TIVS7-2* allele to NTDs has decreased recently.

MATERIALS AND METHODS

Study Population

Blood samples for genetic analysis were collected after obtaining informed consent from 218 individuals with NTDs (120 females and 98 males, born between 1956 and 1995, mean age 16 years at 1/1/96) and their parents. Subjects were identified and samples collected with the assistance of the Irish Association for Spina Bifida and Hydrocephalus, a support group for families with an affected member, and from a register of NTD births in the Dublin maternity hospitals during 1976-1987. Diagnosis of NTD was based on medical records and clinical assessment. The great majority of subjects presented with spina bifida, with only 9 anencephalocele. The 218 mother/father/case triads were further subdivided according to whether a family history of the condition could be ascertained at interview conducted by a clinician: Those triads in which the index case had a sibling, uncle, aunt, or first cousin with an NTD were classified as "familial"; those in which no such affected relatives occurred were classified as "nonfamilial"; and those in which there was inconclusive evidence of NTDs in close relatives remained unclassified.

Controls

Blood samples were collected from 205 women at first antenatal clinic visit at the Coombe Women's, Rotunda, and National Maternity Hospitals in Dublin, and have been reported elsewhere [Kirke et al., 1996; Molloy et al., 1997, 1998].

Mutation Analysis

DNA from whole blood or stored frozen buffy coats was isolated and *TIVS7* genotyping was performed by polymerase chain reaction and allele-specific restriction digestion as described by Morrison et al. [1996]. MTHFR genotyping of the subjects in this study has been reported elsewhere [Shields et al., 1999].

Statistical Analysis

Analysis of allelic association should ideally be based on comparisons between case and control populations drawn from the same genetic background: however, it is often difficult to prove that case and control groups are appropriately matched. For this reason, parental nontransmitted alleles are often used, since these are drawn from the same genetic background as the case alleles. However, in a fetal disorder where maternal alleles may contribute directly to disease these controls may not be entirely appropriate. Weinberg et al. [1998]

recently presented a log-linear modeling approach that allows assessment of both fetal and maternal effects. Accordingly, we adopted this as our principal approach to analyzing the data. The method models the expected count for each of the 15 triad types shown in Table I by fitting a Poisson regression. A dominant effect of the *TIVS7-2* allele was assumed in the model. The model predicts risk associated with carrying the *TIVS7-2* allele (i.e., the risk conferred by a *TIVS7-1/TIVS7-2* or *TIVS7-2/TIVS7-2* genotype compared with a *TIVS7-1/TIVS7-1* genotype), and estimates the allele frequency. The log-linear model of the fetal genotypic effect is equivalent [Weinberg et al., 1998] to the likelihood method of Schaid and Sommer [1994]. We fitted the log-linear model using the POISSON command of the general statistics package STATA [Statacorp, 1997]. Thus, following Weinberg et al. [1998] the POISSON regression included an offset term to allow for the double exposure of the double heterozygote mating, a linear term equal to the number of alleles carried by the mother and father (allowing for parental mating type stratification, assuming Hardy-Weinberg distribution) and optional modeled terms. For example, the 11 family types in Table I in which the case has one or two *TIVS7-2* alleles are coded "1" while the other four are coded "0"; the significance of this case dominant effect is estimated from the model. Relative risks and confidence intervals were calculated directly from the model.

Allele transmission tests were also performed: an exact form of the transmission disequilibrium test [TDT; Spielman et al., 1993] was used to compare the frequency of transmitted and nontransmitted alleles from heterozygous parents. This test is robust to departures from Hardy-Weinberg equilibrium.

It would be appropriate to present one-sided *p*-values to test the prior hypothesis of Morrison et al. [1996] that *TIVS7-2* is a risk factor. However, we chose to present the more conservative two-sided p-values, which tend to reduce the apparent significance of the findings. A formal test of interaction between MTHFR and T genotypes was performed by assigning a control genotype, artificially constructed from the two parental nontransmitted alleles. Analysis of variance of genotypic main effects (homozygosity for MTHFR 677T and carrier status for TIVS7-2) and an interaction term between the two genotypes were modeled to predict case control status.

TABLE I. Frequencies of *T* (*TIVS7*) Genotypes Within NTD Case—Parent Triads*

		Neural tube defect case			
Maternal	Paternal	1/1	1/2	2/2	Total
1/1	1/1	70 (34)	—	—	70 (34)
1/1	1/2	24 (7)	30 (19)	—	54 (26)
1/2	1/1	19 (9)	28 (14)	—	47 (23)
1/2	1/2	7 (2)	10 (6)	7 (3)	24 (11)
1/1	2/2	—	8 (3)	—	8 (3)
2/2	1/1	—	9 (4)	—	9 (4)
2/2	1/2	—	2 (0)	2 (2)	4 (2)
1/2	2/2	—	1 (0)	1 (1)	2 (1)
2/2	2/2	—	—	0 (0)	0 (0)
	Total:	120 (52)	88 (46)	10 (6)	218 (104)

**TIVS7* genotypes are as follows: 1/1 = *TIVS7-1/TIVS7-1;* 1/2 = *TIVS7-1/TIVS7-2;* 2/2 = *TIVS7-2/TIVS7-2.* Pre-1980 cases are in parentheses.

RESULTS

A total of 218 NTD cases and their parents were genotyped; 45.0% of cases carried the *TIVS7-2* allele (i.e., had the *TIVS7-1/TIVS7-2* or *TIVS7-2/TIVS7-2* genotype) compared with 39.4% of mothers and 42.2% of fathers (Table II). The excess in cases reflects the greater number of heterozygotes, since the rarer *TIVS7-2/TIVS7-2* homozygotes have approximately equal frequencies among cases and parents. Examination of families with different genotypic combinations (Table I), indicates that the excess of *TIVS7-1/TIVS7-2* offspring arises mainly from the common class of *TIVS7-1/TIVS7-1* × *TIVS7-1/TIVS7-2* matings, which have an excess of heterozygous outcomes.

Log-linear modeling shows that carrying the *TIVS7-2* allele confers a suggestion of a possible increased risk to cases (relative risk [RR] = 1.36, 95% CI = 0.95–1.94); however, this trend is only approaching statistical significance ($P = 0.096$). Given the known familial risk for NTDs [Little, 1992] and the decline in incidence in Ireland [Johnson et al., 1998] and other countries [Murphy et al., 1996] during the latter part of the twentieth century, we investigated whether the *TIVS7-2* carriers were clustered within familial cases or in cases from before 1980 (the median birth year of our case population), when there was a higher rate of NTDs in Ireland. The allele confers a risk of borderline significance among the cases with no identified family history (RR = 1.59, $P = 0.063$, Table II). A more striking effect was noted when the older cases (born before 1980) were investigated: 50.0% of this group carry at least one *TIVS7-2* allele, and the risk for this group is correspondingly greater (RR = 2.09). The nominal statistical significance for this subgroup is reasonably high ($P = 0.006$), and is still significant even after being multiplied by five to correct for the five tests performed ($P = 0.030$).

To determine whether the age cut-off chosen above reflects the point at which a change in risk occurred, the data was broken down according to the four quartiles of birth date (Table III). The first two quartiles have a higher risk (approximately twofold) than the last two, where the risk appears to be independent of TIVS7 genotype. This suggests that, rather than a gradual temporal trend, a reasonably abrupt change in the incidence of *TIVS7-2* associated disease etiology occurred. However, there is too little data to model the effects of time more accurately than the simple observation that *TIVS7-2* is associated with a higher risk of NTD before 1980. The same calculations for the other identified risk factor in this population, *MTHFR 677T*, does not indicate such strong changes in genotypic risk with time (Table III).

Although log-linear modeling [Weinberg et al., 1998] and related approaches [Schaid and Sommer, 1994] are generally more powerful than the TDT test, the latter is more robust in the presence of departures from Hardy-Weinberg equilibrium. In our complete data set 85 *TIVS7-2* alleles were transmitted to cases from het-

TABLE II. Frequencies of *TIVS7* Genotypes in Different Groups, and Risk Predicted From Log-Linear Analysis of Familial Patterns*

	Genotype frequency			Risk of carrying *TIVS7-2*		
Population	1/1	1/2	2/2	RR	95% interval	*p*
All cases	120	88	10	1.36	0.95–1.94	0.096
Pre-1980	52	46	6	2.09	1.23–3.55	0.006
1980+	68	42	4	0.92	0.56–1.52	0.757
Familial	54	32	5	1.05	0.60–1.84	0.860
Nonfamilial	61	51	5	1.59	0.97–2.60	0.063
Mothers	132	73	13			
Fathers	126	82	10			
Controls	215	70	10			

**TIVS7* genotypes are as follows: 1/1 = *TIVS7-1*/*TIVS7-1;* 1/2 = *TIVS7-1*/*TIVS7-2;* 2/2 = *TIVS7-2*/*TIVS7-2.*

erozygous parents, compared with 70 *TIVS7-1* alleles ($P = 0.26$). Among the pre-1980 cases the transmission bias was more pronounced: 48 *TIVS7-2* alleles were transmitted compared with 26 *TIVS7-1* alleles ($P = 0.010$). Thus, the same overall and historical trends are observed. The allele transmission ratio from heterozygous parents favoring *TIVS7-2* is more pronounced among the pre-1980 group (48:26) and marginally reversed in the younger group (37:44). This difference between the older and younger groups is itself statistically significant ($P = 0.023$, Fisher's exact test). However, it should be noted that the direct comparison between the *TIVS7-2* carriers among older and younger groups (Table II), although showing exactly the same trend, does not reach statistical significance. This may be chance, or may reflect some slight difference in genetic population substructure between the older and more recent cases, since the latter statistic is sensitive to this, whereas the more robust log-linear modeling is not.

The TDT assumes that two risk-conferring alleles contribute more to disease than one, i.e., that a homozygous genotype has twice the effect of a heterozygous genotype. A dominant mode of action of *TIVS7-2* may be more likely. To test this, we calculated a matched genotype relative risk [MGRR, Schaid and Sommer, 1994]. There were 65 cases with one or two *TIVS7-2* alleles, where neither of the nontransmitted parental alleles were *TIVS7-2*, and 50 cases lacking *TIVS7-2* where the nontransmitted alleles included one or two *TIVS7-2* alleles ($P = 0.19$). Among the pre-1980 cases, the frequencies were 36 and 18, respectively, which was significant ($P = 0.02$). Thus, allele transmission tests marginally favor the dominant model over the allele dosage model tested by the TDT.

Further inspection of the genotypic combinations observed in families (Table I) reveals no additional striking patterns. The a priori model of *T* gene action is within the embryo. Nevertheless, it is conceivable that parental genotypes might influence disease, either through direct effects of expression of the maternal genes, or through genomic imprinting. Such effects would be reflected in biases such as a difference in outcome from reciprocal *TIVS7-1*/*TIVS7-1* homozygote × *TIVS7-1*/*TIVS7-2* heterozygote matings. There are more *TIVS7-1*/*TIVS7-1* outcomes of the double heterozygote matings than would be expected given the overall trend, but this is likely to be a chance finding, and there is no obvious biological model to explain such an outcome. A formal test for a maternal gene effect was performed using the log-linear model. There was no evidence for a significant dominant or recessive maternal contribution (RR = 0.91; $P = 0.59$ and RR = 1.17; $P = 0.64$, respectively).

The distribution in the NTD cases of the three MTHFR genotypes in relation to the three *TIVS7* genotypes is shown in Table IV. Among the cases carrying the *TIVS7-2* allele there is no greater frequency of *MTHFR-TT* homozygotes than among the cases who are homozygous for *TIVS7-1* ($P = 0.40$, 95% CI = 0.37–1.49). Thus, a strong interaction of these two genotypes is unlikely to influence NTDs in this population. The formal analysis of variance test of interaction between the two genotypes was not significant ($P = 0.58$). This was also true among the subset of pre-1980 cases, which had a higher frequency of the *TIVS7-2* allele. This suggests that the biological mechanisms whereby these two genetic risk factors contribute to the etiology of NTDs may be independent.

The *TIVS7* genotype frequencies in 205 controls are shown in Table II: 21.9% of the alleles were *TIVS7-2*, and the genotype frequencies are extremely close to Hardy-Weinberg equilibrium. The allele frequency estimated from the log-linear model of a case dominant genotypic effect is 21.0%, which indicates that parental nontransmitted allele frequencies are close to those of the control population. When the allele frequency is estimated by the log-linear model for the pre-1980 group alone, it is 18%, indicating that sampling of families with cases born pre-1980, and in 1980 and later, has not been subject to any appreciable subpopulation bias. The frequency of genotypes with one or two *TIVS7-2* alleles is 39.0% in the controls, compared with

TABLE III. Genotypic Risk of Carrying Brachury *TIVS7-2* and of Homozygosity for *MTHFR 677T* Predicted From Log-Linear Modeling by Period of Birth

Date of birth	Genotype	RR	95% CI
Quartile 1: 1956 to 1976	*TIVS7-2* carrier	2.08	1.01–4.28
	MTHFR-TT	0.98	0.44–2.16
Quartile 2: 1976 to 1980	*TIVS7-2* carrier	2.03	0.97–4.25
	MTHFR-TT	1.54	0.66–3.56
Quartile 3: 1980 to 1984	*TIVS7-2* carrier	0.81	0.40–1.67
	MTHFR-TT	2.34	1.06–5.17
Quartile 4: 1984 to 1995	*TIVS7-2* carrier	1.02	0.50–2.08
	MTHFR-TT	1.73	0.67–4.50

TABLE IV. Frequency of *T* (*TIVS7*) and *MTHFR* (*C677T*) Genotype Combinations Among NTD Cases

	MTHFR (*C677T*)[b]			
T (*TIVS7*)[a]	CC	CT	TT	Total
All cases				
11	42	53	25	120
12	34	39	15	88
22	6	3	1	10
Total	82	95	41	218
Pre-1980 cases only				
11	15	28	9	52
12	15	22	9	46
22	2	3	1	6
Total	32	53	19	104

[a]*TIVS7* genotypes are as follows: 1/1 = *TIVS7-1/TIVS7-1;* 1/2 = *TIVS7-1/TIVS7-2;* 2/2 = *TIVS7-2/TIVS7-2.*
[b]*MTHFR C677T* genotypes are as follows: CC = homozygous wild type; CT = heterozygote; TT = homozygous thermolabile.

45.0% in the cases (Table I; OR = 1.28; CI = 0.87–1.87; $P = 0.22$).

DISCUSSION

Historically, Ireland has had one of the highest incidences of NTDs, with rates as recently as the 1970s being approximately six per thousand (P.N. Kirke, unpublished data). During the past 20 years there has been a steady decline in this rate in the Dublin region from 4.7 per thousand in 1980 to 1.2 per thousand in 1994 [Johnson et al., 1998]. The legal and social constraints on prenatal screening and elective abortion that have prevailed in Ireland up to the present time preclude the trivial explanation that the reduced incidence of NTDs is attributable to increased detection and termination of affected pregnancies. It is therefore clear that the underlying biological factors that contribute to NTD pathogenesis must have changed. It is likely that one of the important factors to have changed is an improvement in dietary folate intake, brought about by an increase in the year-round availability of folate-rich foods and by folic acid supplementation of cold cereals and other foods. Nevertheless, the rate of NTD births remains at around one per thousand in most industrialized countries, and consequently the search for both folate-dependent and folate-independent etiologic factors is ongoing.

There are now in excess of 40 mutations in the mouse that are known to cause a variety of NTD and midline defect phenotypes [Harris and Juriloff, 1997]. Some of these can be partly rescued by folate administration, for example, those involved in null mutations of *Pax3* [Fleming and Copp 1998] and *Cart1* [Zhao et al., 1996], and others such as the curly tail, *ct*, mutant are folate independent. The human homologues of these developmentally important genes are excellent candidate susceptibility loci for human NTDs.

Accordingly, we have further investigated the recent report of an association between the *TIVS7-2* allele at the *T (Brachyury)* locus and susceptibility to NTD in a mixed U.K. and Dutch study population [Morrison et al., 1996] by examining the incidence of this variant in a large cohort of Irish NTD cases. Mutations in the *T* transcription factor in both mice and zebrafish act in a dose dependent fashion and lead to a failure of notochord formation and abnormal development of the posterior axial mesoderm [Herrman and Kispert, 1994]. Thus, the consequences of T dysfunction in embryogenesis are not species restricted and are therefore likely to be fundamental in nature. T functions in mesoderm differentiation by activating gene transcription but little is known of its target genes. However, there is some evidence that T interacts directly with the promoters of the embryonic FGF (*eFGF*) and *Bix1* genes. The latter encodes a protein that appears to direct cells along alternative mesoderm/endoderm pathways [Tada et al., 1998; Casey et al., 1998].

In the 218 NTD case-parent triads available to us we observed a trend toward an excess of the *TIVS7-2* allele among the cases (OR = 1.36) that was stronger in those born before 1980 (OR = 2.09, corrected $P = 0.030$). This confirmation of the observations of Morrison et al. [1996] establishes the above *T* gene variant as a modest but important risk factor in NTDs. As the *TIVS7-2* allele is defined by a polymorphism in intron 7 of the gene, it is likely either that the biological consequence of the variant is in modified RNA splicing or that the variant is a marker for a control or structural polymorphism with which it is in linkage disequilibrium. A mutation mandating a Gly to Asp polymorphism at residue 177 in the DNA binding domain of the human T protein has recently been described [Papapetrou et al., 1997]. This variant is potentially functional since it appears to affect the formation of the T dimer, but genetic studies in NTD families have not supported an association with susceptibility to NTD [Morrison et al., 1999]. Some association with NTD might have been expected if the "destabilizing" Asp allele were in linkage disequilibrium with the *TIVS7-2* allele; however, *TIVS7-2* occurs more commonly on chromosomes carrying the Gly177 allele [Morrison et al., 1998].

Our observation is that the *TIVS7-2* allele is a significant risk factor for NTD in cases born prior to 1980, but does not appear to have contributed to NTD outcome in cases born between 1980 and the present. It consequently implies that the risk conferred by this *T* locus variant has diminished with time and may, in fact, no longer apply to the majority of carriers. This contrasts with the homozygous thermolabile MTHFR genotype, which appears to have remained an NTD risk factor to date (Table III).

Our data suggest that the risk conferred by *TIVS7-2* does not act synergistically with the risk conferred by the homozygous thermolabile MTHFR genotype since *TIVS7-2* carriers are no more likely to have this genotype than noncarriers. As individuals who are homozygous for the thermolabile *MTHFR* allele have a tendency toward reduced levels of serum folate [Harmon et al., 1996], this genotype would be expected to potentiate other folate-dependent genetic effects. The absence of evidence in support of such an etiologic interaction suggests that the risk associated with the *TIVS7-2* allele may, in fact, be independent of folate. If this is so, the recent reduction in the incidence of NTDs in Ireland may not be entirely due to an improvement in the folate status of the general population. Whereas

changes in neonatal treatment of spina bifida may have resulted in a slight increase in survival so that some of the more recent cases are more severe, this is unlikely to have resulted in a marked decline in *TIVS7-2* associated risk.

Based on the findings of this study, it is apparent that there are at least two independent genetic factors that can compromise closure of the neural tube, and that are causative agents in NTDs. Polymorphic variants of the *T* locus product, a developmentally important transcription factor, and MTHFR, an enzyme critical for homocysteine metabolism, may act through different mechanisms. Clearly there is a strong need for studies in other populations, although complex temporal and geographical changes in risk factors may require many studies to establish a clear picture.

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